Heat Resistance and Fatty Acid Composition of *Listeria monocytogenes:* Effect of pH, Acidulant, and Growth Temperature†

ABSTRACT

The objective of this study was to determine the influence of pH, acidulant, and growth temperature history on the heat resistance and fatty acid composition of *Listeria monocytogenes* Scott A. Cells were grown to late exponential phase (OD_{600} = 0.6) at 10, 19, or 37°C in brain heart infusion broth acidified to pH 5.4 or 7 with either acetic or lactic acid. Thermal death times at 60°C subsequently were determined by using a submerged-coil heating apparatus. The surviving cell population was enumerated by spiral-plating heated samples onto tryptic soy agar supplemented with 0.6% yeast extract and 1% sodium pyruvate. The thermal resistance of cells cultured at a particular temperature was significantly lower ($P < 0.05$) when lactic acid was used to acidify the medium to pH 5.4. Regardless of acid identity, *D* values significantly decreased ($P < 0.05$) with increased growth temperature when the pH of the growth medium was 5.4, whereas *D* values significantly increased ($P < 0.05$) with increased temperature at pH 7. At pH 5.4 adjusted with lactic acid, *D* values were 1.30, 1.22, and 1.14 min for cells grown at 10, 19, and 37°C, respectively. At pH 5.4 adjusted with acetic acid, *L. monocytogenes* failed to grow at 10°C; the *D* values were 1.32 and 1.22 min when the cells were grown at 19 and 37°C, respectively. At pH 7, the *D* values were 0.95, 1.12, and 1.28 min with lactic acid and 0.83, 0.93, and 1.11 min with acetic acid at 10, 19, and 37°C, respectively. The most abundant fatty acids (44 to 82%) were branched-chain saturated fatty acids (anteiso- and iso-C15:0 and iso-C17:0) regardless of pH, acidulant, or growth temperature. However, there was an increase in C15:0 isomers at the expense of iso-C17:0 when the growth temperature was lowered from 37 to 10°C. While variable changes in longer-chain fatty acids were found, the percentage of longer-chain (C16 and C18) fatty acids was greatest when *L. monocytogenes* was grown at 37°C regardless of pH or acidulant. This study demonstrates that the heat resistance of *L. monocytogenes* depends upon its growth conditions.

The heat resistance of microorganisms is documented to be affected not only by environmental conditions such as growth temperature, medium composition, and stage of growth, but also by conditions during and after heating, such as composition and pH of the heating menstruum and methodology used for detection of survivors *(27).* Bacterial cells grown at higher temperatures are known to be more heat resistant than those grown at lower temperatures *(16, 17, 23, 28).* There have been several reports that the reduced heat resistance of cells grown at low temperatures may be due to an increase in the concentration of unsaturated fatty acids in the cytoplasmic membrane, which increases membrane fluidity, reduces viscosity, and thus decreases thermotolerance *(7, 11, 18).*

Food preservation factors, such as temperature, water activity, pH, etc., constitute environmental stresses that can alter an organism's susceptibility to subsequent stress conditions. For example, sublethal heat stress renders an organism more resistant to subsequent heat treatment that would otherwise be lethal. Cross protections also can occur, in that exposure to one stress alters resistance to another. For example, acid pH exposure can induce other stress responses including resistance to thermal stress *(6, 14).* This example indicates that the bacterial cell has a limited number of basic systems for eliciting gene expression; changes induced by one stress would protect cells against other environmental challenges. The mechanism of cross responses, known as global stress response, has received recent attention because of its implications for the safety of milder preservation technologies.

In a study by Farber and Pagotto *(6),* when *L. monocytogenes* cells grown for 24 h at 30°C in tryptic soy broth were acid shocked with HCl, thermotolerance at 58°C was significantly ($P < 0.05$) increased compared to that of the control cells that were not exposed to acid treatment before heating. While this study provided some rational for the acid shock-induced cross protection against heat lethality in *L. monocytogenes,* there appears to be no work reported on the effect of acidic growth conditions (acid adaptation) in combination with growth temperature on the organism's susceptibility to heat. Accordingly, the work reported herein was undertaken to determine the influence of pH, acidulant, and growth temperature history on the heat resistance and fatty acid profile of *L. monocytogenes* Scott A. Because fatty

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acid composition regulates membrane fluidity, changes in fatty acid composition of *L. monocytogenes* were considered important as one possible way of understanding the biochemical basis for the unusually high heat resistance of this organism.

MATERIALS AND METHODS

Bacterial strain. *Listeria monocytogenes* Scott A obtained from the ERRC culture collection was used in the study. Cells were grown on brain heart infusion agar (BHIA, pH 7.4; Difco Laboratories, Detroit, Mich.) slants at 37°C for 24 h, and held at 4°C. The cultures were transferred periodically to maintain viability. Brain heart infusion broth (BHIB pH, 7.4 ± 0.2; Difco) was used to grow cultures before all experiments. Peptone (0.1%, wt/vol) was used as a diluent for cultures grown in BHIB.

Preparation of test broth. Brain heart infusion broths (BHIB, 100 ml) in 250-ml Erlenmeyer flasks were adjusted with appropriate amounts of 1 M stock solutions of lactic or acetic acid to pH 7 or 5.4 (measured with a pH meter and combination pH electrode) (Corning pH meter model 340, Corning Combination 3-in-1 Electrode, Corning, N.Y.) and sterilized for 15 min at 121°C. The pH of the BHIB was checked again following sterilization and equilibration to room temperature on a portion of the broth and, if necessary, pH adjustments to the broths were made with sterile solutions of the acids under a laminar-flow biological safety cabinet. The volume of acids added to achieve pH 7 or 5.4 was noted and used to calculate the concentration of each acid in broth at each test pH. The concentrations of acetic and lactic acids in broths at pH 7 were 0.012 and 0.008 M, respectively; at pH 5.4, the concentration of either acetic or lactic acid was 0.027 M. The acidified broth was inoculated with 0.1 ml of an 18-h *L. monocytogenes* culture and then incubated at 37, 19, or 10°C on a rotary shaker (120 rpm). Cells were incubated to an absorbancy at 600 nm of 0.6, obtained with a Shimadzu UV-160 recording spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, Md.). Uninoculated broths at each pH were used as blanks. Two replications in duplicate were performed for each treatment.

Thermal inactivation studies. The broth suspensions were heated at 60°C using a submerged-coil heating apparatus *(4)*. It comprises a stainless-steel coil fully submerged in a thermostatically controlled water bath which allows microbial suspensions to be heated to between 20 and 90°C with a short time to temperature equilibrium. During the heating procedure, samples (0.2 ml) were removed at predetermined time intervals. Where low cell numbers were expected, 0.6-ml aliquots were removed. Samples were cooled rapidly to room temperature in peptone (0.1%, wt/vol) water. Survivors were diluted serially in peptone water and plated onto tryptic soy agar (Difco) supplemented with 0.6% yeast extract and 1% sodium pyruvate. Plates were incubated at 30°C for 4 days before the colonies of survivors were counted.

Determination of *D* values. *D* values (min) were determined by plotting the $\log_{10}$ number of survivors against time for each heating temperature. The line of best fit for survivor plots was determined by linear regression, and the negative reciprocal of the slope was used to calculate the *D* value in minutes for each temperature. Only survivor curves with more than five values in the straight portion, with a correlation coefficient (r^2) >0.90, and descending more than 5 log cycles were used.

Statistical analysis. The heat-resistance data were analyzed by analysis of variance (ANOVA) using SAS software *(25)* to determine if there were statistically significant differences among the treatments. The Bonferroni mean separation test was used to determine significant differences ($P < 0.05$) among means *(21)*.

Fatty acid analysis: harvesting and concentration of cells. *L. monocytogenes* cells were harvested by three successive centrifugations at 17,310 × *g* for 10 min at 2 to 4°C with sterile distilled H_2O washes between centrifugations. The cell pellets were resuspended in sterile distilled H_2O and concentrated by lyophilization in a Labconco Freeze dryer (Model 77530; Labconco Corporation, Kansas City, Mo.). Lyophilized samples were stored at 4°C before analyses. Duplicates of lyophilized samples for each incubation temperature were subjected to lipid extraction.

Lipid extraction and methanolysis. Lipids present in the dried biomass were extracted and converted to fatty acid methyl esters (FAME) using a modification of the methanolysis procedure described by Minnikin et al. *(22)*. Approximately 20 to 40 mg of lyophilized *L. monocytogenes* cells were transferred to a 10-ml glass centrifuge tube with a Teflon screw cap (Thomas Scientific, Swedesboro, NJ) and 3 ml of dry methanol-toluene-trifluormethanesulfonic acid (30:15:1) mixture added. The reaction mixture was heated at 60°C for 16 to 18 h and cooled to room temperature; 2 ml of hexane was added and the mixture vortexed for 1 min. The phases were allowed to separate and the upper hexane layer was transferred to a small column of basic alumina (750 mg, 150 mesh, 5.8 nm) (Aldrich Chemical Co., Inc., Milwaukee, Wis.) prepared in a jumbo glass-wool plugged Pasteur pipette, previously washed with 2 to 3 ml of methylene chloride. The hexane eluate was collected in a 3.7-ml vial. The reaction mixture was reextracted with hexane (1 ml) and the upper layer pipetted onto the column. The combined eluates were evaporated under nitrogen at 20°C, the residue weighed for percent (by weight) lipid determination, and then dissolved in isooctane (0.10 ml) containing methyl heneicosonoate (C21:0, 1.08 mg/ml) as an internal standard for gas-liquid chromatographic analysis (GLC).

Fatty acid analyses. Fatty acid methyl esters (FAME) were analyzed on a Hewlett-Packard (HP, Wilmington, Del.) Model 5890 gas chromatograph equipped with a split capillary injector and a flame ionization detector. Separations were obtained using a HP-Innowax fused silica capillary column, 30 m by 0.53 mm, internal diameter. The carrier gas was He (linear velocity methane, 75 $cm^3\ s^{-1}$) at a split ratio of 15:1. The following oven temperature program was used: initial temperature 120°C for 2 min; then 5°C/min to 230°C; then hold for 10 min. Signal analysis was accomplished by routing the detector output to a Hewlett-Packard Model 3396A integrator and a Hewlett-Packard Model 9122C mass storage unit for subsequent statistical analysis. FAME assignments were made by comparison with standards (NuCheck Prep, Elysian, Minn.) or from plots of log retention time versus carbon number *(26)*.

RESULTS

Heat resistance. The *D* values of *L. monocytogenes* grown to late exponential phase (OD_{600} = 0.6) at 10, 19, or 37°C in brain heart infusion broth acidified to pH 5.4 or 7 with either acetic or lactic acid are in Table 1. Regardless of acid identity, *D* values significantly decreased ($P < 0.05$) with increased growth temperature when the pH of the growth medium was 5.4; *D* values significantly increased ($P < 0.05$) with increased temperature at pH 7. At pH 5.4

TABLE 1. *Effect of pH, acidulant, and the growth temperature on the D values of Listeria monocytogenes at 60°C*

Initial pH	Acid	Mean *D* value[a] at temperature (°C)		
		10	19	37
5.4	Lactic	1.30AB	1.22BC	1.14CD
	Acetic	NG[b]	1.32A	1.22BC
7	Lactic	0.95E	1.12D	1.28AB
	Acetic	0.83F	0.93E	1.11D

[a] Means (two replications) with no letter in common are significantly different ($p < 0.05$).
[b] NG, no growth.

adjusted with lactic acid, *D* values ranged from 1.30 min for cells grown at 10°C to 1.14 min for cells grown at 37°C. At pH 5.4 adjusted with acetic acid, *L. monocytogenes* cells failed to grow at 10°C; the *D* values were 1.32 and 1.22 min when the cells were grown at 19 and 37°C, respectively. *D* values of cells cultured at a particular temperature were significantly lower ($P < 0.05$) when lactic acid was used to acidify the medium to pH 5.4. At pH 7, *D* values were 0.95, 1.12, and 1.28 min for cells grown at 10, 19, and 37°C, respectively, with lactic acid; the values ranged from 0.83 min for cells grown at 10°C to 1.11 min for cells grown at 37°C with acetic acid.

Fatty acid composition. Growth to an OD_{600} of 0.6 of *L. monocytogenes* at 10, 19, or 37°C in brain heart infusion broth acidified to pH 5.4 or 7 with either acetic or lactic acid resulted in qualitative and quantitative alterations in the cell membrane fatty acid composition (Table 2). The most abundant fatty acids were the anteiso- and iso-C15:0 and iso-C17:0 branched-chain fatty acids, with the anteiso-C15:0 isomer more prevalent than the iso- branched-chain isomers. This pattern was observed regardless of pH, acidulant, or growth temperature. The C15:0 to C17:0 fatty acid ratio was dependent on the growth temperature and not on the acidulant or pH. With a decrease in temperature there was an increase in the anteiso- and iso-C15:0 isomers and a concomitant decrease in the iso-C17:0 isomer (Figure 1); at 10°C, C15:0 isomers ranged from 82.1 to 77.8% (by weight); the percentages were 56.7 to 43.9% at 37°C. The iso-C17:0 isomer ranged from 2.2 to 5.3% in cells grown at 10°C and the levels increased to as high as 14.1% at the 37°C growth temperature (Figure 1). Fatty acids C12:0, iso-C13:0, anteiso-C13:0, anteiso-C14:0, and C14:0 were found in minor amounts, i.e., <1.5% (Table 2). The only exception was anteiso-C13:0 where 2.3% was detected under lactic acid-pH 5.4-10°C growth conditions. The anteiso-C16:0 isomer was not detected under any growth conditions at 19°C (Table 2). The normal-chain saturated fatty acids C16:0 and C18:0 increased slightly with increased temperature; the largest amount of these fatty acids (7.6%) was detected under the acetic acid-pH 5.4-37°C growth condition.

There were similar variations in the types and amounts of unsaturated fatty acids (C16:1, C18:1, 18:2, and 18:3). While C18:3 was not detected under all growth conditions at 19°C, the overall sum of unsaturated fatty acids ranged from a low of 2.6% in cells grown at 10°C to a high of 12.6% at 37°C. Surprisingly, unidentified fatty acids with retention times greater than C18:3 were present in cells grown under all conditions tested, with a percentage as high as 21.5% in the growth conditions of acetic acid-pH 7-37°C (Table 2).

TABLE 2. *Effect of pH, acidulant and growth temperature on the fatty acid composition of* Listeria monocytogenes *Scott A*

Retention time (min)	Fatty acyl residue[c]	Fatty acyl residue (wt %[a]; mean, $n = 2$) of *L. monocytogenes* cells grown under acidulant[b]/pH/temperature										
		LA/5.4/10	LA/7/10	AA/7/10	LA/5.4/19	AA/5.4/19	LA/7/19	AA/7/19	LA/5.4/37	AA/5.4/37	LA/7/37	AA/7/37
8.86	C12:0	0.2	–[d]	–	–	–	0.2	–	–	0.2	0.1	0.1
9.89	i-C13:0	0.4	–	0.2	–	–	1.3	–	0.2	0.2	0.2	0.2
10.19	ai-C13:0	2.3	1.2	0.5	0.3	0.4	0.4	0.2	0.1	0.1	0.1	0.1
11.91	ai-C14:0	0.8	–	1.4	0.6	0.8	0.9	0.7	0.2	0.4	0.3	0.3
12.89	C14:0	0.3	–	0.2	0.3	0.2	1.0	0.2	0.2	0.4	0.5	0.4
13.93	i-C15:0	8.5	12.4	13.3	9.9	14.9	16.9	13.7	13.8	10.9	8.8	7.1
14.26	ai-C15:0	72.4	69.7	64.5	68.4	64.3	56.3	66.1	39.0	45.8	35.1	38.4
15.90	i-C16:0	0.5	–	–	1.0	1.1	0.8	0.7	–	2.3	0.7	0.7
16.33	ai-C16:0	0.2	–	0.3	–	–	–	–	3.0	–	0.5	0.4
16.84	C16:0	1.6	1.0	2.5	0.6	0.6	2.2	0.5	4.6	5.8	3.1	2.40
17.38	C16:1	0.8	0.4	1.8	0.9	1.9	3.0	2.2	7.8	6.4	6.4	8.7
18.13	i-C17:0	5.3	4.6	2.2	15.0	12.3	8.6	12.0	12.5	11.5	14.1	13.5
20.49	β-OH-C16:0	1.4	1.0	1.4	0.9	0.9	1.1	0.9	0.9	1.3	1.0	0.7
20.59	C18:0	1.3	1.1	0.8	0.3	0.1	0.6	0.2	2.1	1.8	2.4	2.5
20.98	C18:1	1.2	1.4	0.8	0.5	0.3	0.6	0.2	2.0	2.7	2.9	1.4
21.83	C18:2	0.4	–	–	0.2	0.2	0.2	–	0.7	0.9	1.3	0.2
23.01	C18:3	0.2	0.8	0.8	–	–	–	–	1.9	1.2	1.5	1.5
25.97	C21:0											
	Unknown	2.2	6.3	9.3	1.1	1.90	6.2	2.5	11.0	7.9	21.0	21.5

[a] Area under curve of GLC printout weight %, ±0.1%.
[b] AA, acetic acid; LA, lactic acid.
[c] Fatty acyl residue: i, iso; ai, anteiso.
[d] Species absent or below detection limit.

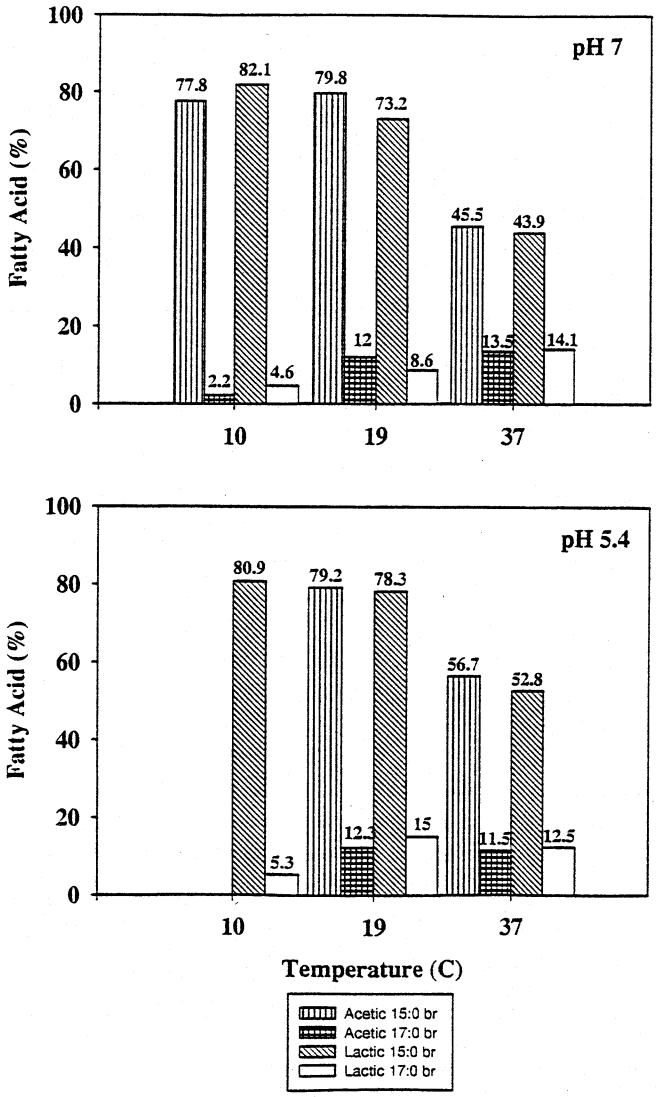

FIGURE 1. *Percentages of branched-chain fatty acids found in* Listeria monocytogenes *grown at 10, 19, or 37°C in brain heart infusion broth, pH 7 or 5.4, adjusted with acetic or lactic acid.*

DISCUSSION

This study assessed the heat resistance of *L. monocytogenes* grown to late exponential phase (OD_{600} = 0.6) at 10, 19, or 37°C in brain heart infusion broth acidified to pH 5.4 or 7 with either acetic or lactic acid. Also studied was the effect of these treatments on the cell membrane fatty acid composition. Bacterial response to heat depends upon the physicochemical state of the membrane as well as the heating environment. Previous reports have shown correlations between bacterial membrane fatty acid composition and heat resistance *(1, 10, 13). Vibrio parahaemolyticus* grown at different temperatures and at different sodium chloride concentrations showed alterations in membrane fatty acid profile that correlated with an altered response to subsequent heat treatment *(1).* Hansen and Skadhange *(11)* demonstrated an altered heat resistance along with altered membrane fatty acid profiles in *Escherichia coli* grown at different temperatures.

In the present study, alterations in fatty acid profiles were accompanied by an increase in the heat resistance of cells grown in BHI broth adjusted to pH 7 with acetic acid or lactic acid. Increased levels of anteiso- and iso-C15:0 isomers were accompanied by decreased levels of iso-C17:0 in cells as the growth temperature decreased. These changes were associated with lower heat resistance. This trend in the alteration of major fatty acids and the heat resistance of *L. monocytogenes* is in agreement with reports in the literature. Juneja and Davidson *(12)* grew *L. monocytogenes* Scott A in tryptose phosphate broth to an OD_{600} of 0.6 at temperatures ranging from 7 to 35°C and reported that branched-chain C15:0 fatty acids decreased while branched-chain C17:0 fatty acids increased with increasing growth temperature. Lowering the growth temperature has been demonstrated to result in cells with lower-melting point fatty acids that help to maintain membrane fluidity and such cells are more sensitive to heat *(10).* Cells of *L. monocytogenes* grown at 19°C had a *D* value at 60°C of 0.8 min when heated in a sausage slurry, while cells grown at 37°C had a *D* value at 60°C of 1.6 min *(2). L. monocytogenes* growing on refrigerated meat generally are inactivated by heat more efficiently and at lower temperatures, provided that the bacteria will proliferate on raw, refrigerated meat *(3).*

In contrast, the heat resistance of cells grown in BHI broth acidified to pH 5.4 with either acid increased as the growth temperature decreased. This opposite trend was observed even though the fatty acid profile of these cells was modified at lower temperatures in the direction of lower-melting point fatty acids, as was observed in the case of cultures grown at pH 7. The higher heat resistance of *L. monocytogenes* cultures grown in BHI broth acidified to pH 5.4 at low temperatures might be due to the induction and repression of the synthesis of stress proteins in these cultures. In a study by Davis et al. *(5),* when total cellular proteins of *L. monocytogenes* cultures adapted at pH 5.8 were isolated and analyzed by two-dimensional gel electrophoresis, expressions of at least 23 proteins were affected; of these, 11 were induced and 12 repressed. While no information is available to indicate possible roles of these proteins in heat tolerance, it is tempting to speculate that they may protect cells against subsequent heat lethality. Such cross-protection experiments with *L. monocytogenes* were carried out by Farber and Pagotto *(6).* In that study, heat resistance of the organism at 58°C in whole milk was significantly increased ($P < 0.05$) when cells were acid shocked with HCl before heating. In this study, the heat resistance of cells grown at pH 5.4 decreased as the growth temperature increased. These findings might be explained by the failure of full induction of stress proteins such as heat shock proteins (like GroEL or DnaK) which may be essential for thermotolerance. Nevertheless, the parallel decrease in heat resistance of cells grown at pH 5.4 with increasing temperature suggests an involvement of general stress proteins in the induction of thermotolerance and cross protection.

It has been shown that an organism in response to a range of environmental stresses, such as temperature, ethanol, viral infection, methylating agents, antibiotics, amino acid restriction, etc., produces a rapid expression of a distinct set of polypeptides, the heat shock proteins (HSPs) or stress proteins *(8, 15).* Generally speaking, all stresses to

some extent cause protein denaturation and increase the concentration of unfolded proteins in the cell. Such an event may be the trigger for inducing the signals for HSP synthesis *(24)*. Although the actual mechanism by which these stresses contribute to induced thermotolerance has not been fully elucidated, it is thought that a sigma factor is responsible for the induction of heat shock proteins *(9)*.

Environmental conditions present during growth, such as pH, atmosphere, temperature, growth medium, etc., have been demonstrated to be responsible for qualitative and quantitative changes in the membrane fatty acid profile of bacteria and have been attributed to adaptations *(20)*. High-melting point fatty acids (saturated, long- and normal-chain fatty acids) decrease membrane fluidity *(19)*, whereas low-melting point fatty acids (i.e., unsaturated and branched-chain fatty acids) increase membrane fluidity *(29)*. Since *L. monocytogenes* can survive and grow at refrigeration temperatures, the organism must adapt to these particular conditions. On the basis of the results of this study, it could be argued that the observed increase in anteiso- and iso-C15:0 isomers at the expense of iso-C17:0 helps the bacterial cell to maintain its membrane fluidity at low temperature.

Our results indicate that sensitivity of *L. monocytogenes* cells to heat is increased at low temperatures provided the pH during growth is 7; the cells grown at low temperatures become more heat resistant if the pH during growth is 5.4. This work could have implications in thermal inactivation modeling studies; *L. monocytogenes* must be in its most resistant state for thermal death data acquisition. Also, food processors should take into account the environmental conditions during growth while designing thermal processes that ensure safety against this organism in ready-to-eat foods.

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